REPRODUCTION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS IN CHICK EMBRYO FIBROBLAST SUSPENSIONS

[Following is the translation of an article by A. S. [illegible] Novokhatskiy and L. N. Mishin, Institute of Virology imeni D. I. Ivanovskogo, AMN USSR, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology), No 5, 1968, pp 566--574. It was submitted on 20 Nov 1967.]

The missions of practical virology require the further study and development of simple and sufficiently effective methods for the cultivation of viruses. These requirements are answered to a known degree by the method, developed after the discovery of the principle of fermentation dispersion of tissue, of cultivation of viruses in suspensions of trypsinized cells [5, 7, 11, 14]. Suspensions of primarily trypsinized fibroblasts of chick embryos were used with success for the cultivation of viruses of fowl plague, tick-borne encephalitis, Newcastle disease, vesicular stomatitis, Aueski vaccine, and equine encephalomyelitis [12, 15, 14].

We studied the peculiarities of multiplication of virus in suspension on a model of VEE - fibroblasts of chick embryos, using a unit, which was developed in our institute, for the deep cultivation of cells. This unit makes it possible to aerate the culture with a mixture of air with a set percentage of carbon dioxide.

Materials and Methods

The cells, fibroblasts of chick embryos (FCE), were obtained with the help of the conventional method of trypsinization from 10—11 day old chick embryos. As a rule the cells were diluted in a medium of the following composition: 0.5% solution of lactalbumin hydrolyzate (45%), medium No 199 (45%), heated bovine serum (5—10%), and 100 AU/ml each of penicillin and streptomycin.

We used the virus of Venezuelan equine encephalomyelitis (VEE) which had preliminarily undergone a number of passages in monolayer cultures of fibroblasts of chick embryos.

The culture was infected by means of introduction into the prepared cellular suspension of specific amounts of an appropriate dilution of virus-containing fluid in Hanks solution. In the tests for studying the dynamics of accumulation of virus we used the following method: the suspension of cells was centrifuged for 10 minutes at 1500 rpm, the supernatant fluid was removed, and the cells were

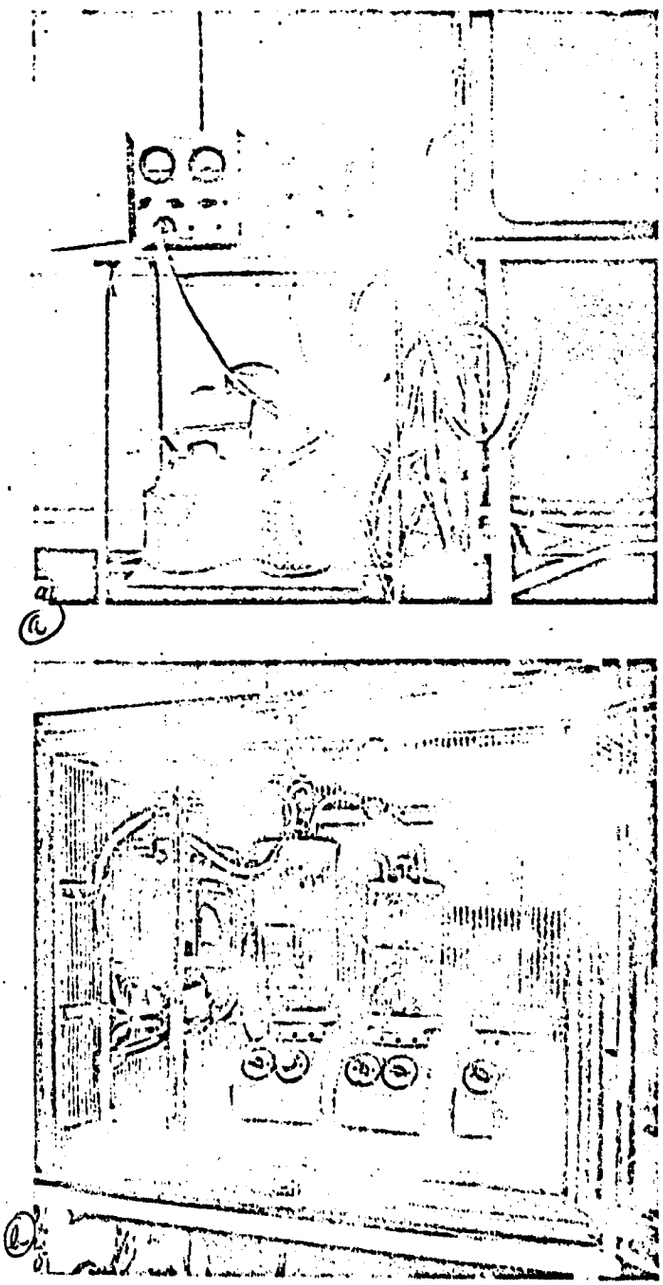

Fig. 1. General view of unit for deep cultivation.
a - units for automation and control; b - interior view of incubator.

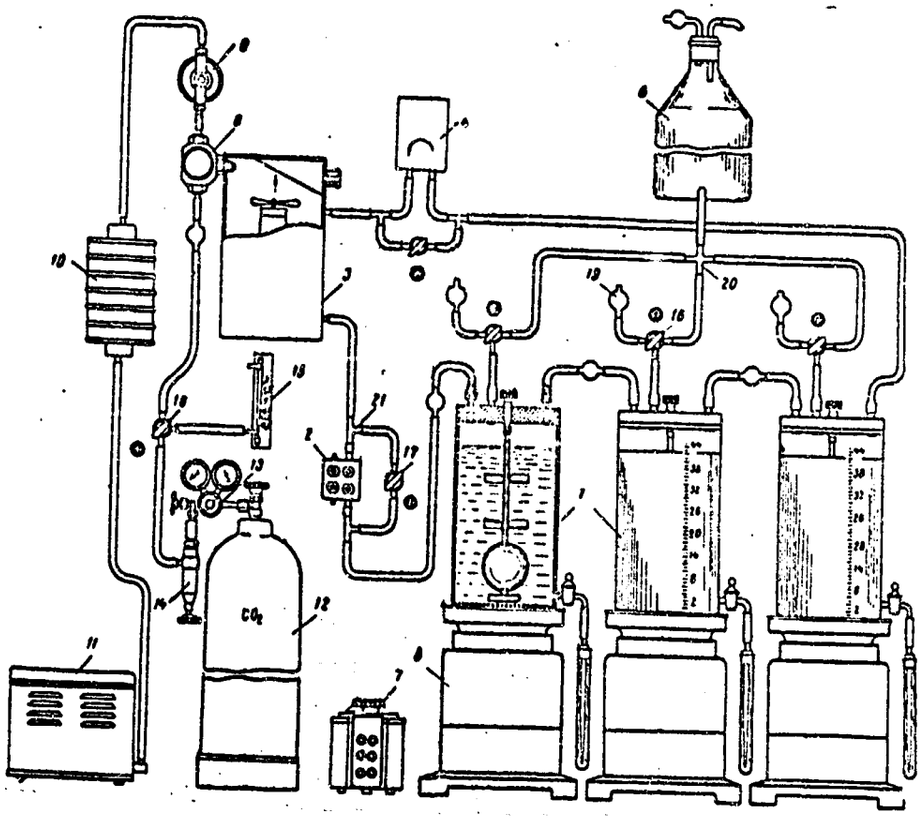

Figure 2. Main arrangement of the unit for deep cultivation.

resuspended in virus-containing fluid. After contact for 30 minutes at 37° the cells were again precipitated by centrifuging, washed three times with Hanks solution, and diluted in fresh nutrient medium up to the required density. A sample taken at this moment was taken as 0 hours.

In the work we used the semi-automatic system for suspension cultivation of cells of the batch type (Fig. 1). The main arrangement of the unit is shown in Fig. 2.

The culture vessels were beakers (1) made from molybednum glass which were equipped with a connecting piece with a tap for decantation of suspension and a fluoroplastic cover with connecting pieces, two of which serve for switching in the circulating system of aeration, and the third for the sterile admission of fresh nutrient medium from vessel 5.

For mixing the suspension a floating paddle magnetic stirrer made from molybdenum glass is used. The lifting force of the spherical float presses the upper end of the stirrer shaft to a conical screw mounted in the center of the cover which fulfills the role of a thrust bearing. To the lower portion of the float is welded a ferromagnetic rod which is armored with glass. The stirrer is revolved from a standard magnetic driving gear, while both rotation and centering of the lower end of the stirrer relative to the vessel is ensured by the magnetic field. A distinctive feature of the stirrer is the absence of rubbing parts in the liquid with effective mixing, which is conditioned by the presence of the paddles. For aeration of the suspension a hermetic circulating system of the closed type is used. It ensures the continuous flow of sterile air with a set concentration of CO_2 over the surface of the suspension. Circulation of the gaseous mixture is ensured by an air micropump (4) with a regulatable capacity from 0.1 to 2 l/min. The required concentration of CO_2 is ensured by an apparatus of automatic regulation, the pickup of which is included in the aeration system. Preliminary establishment of the required percentage of CO_2 is done with the help of a special regulator within limits from 1 to 10%. All the elements of the apparatus are calculated for prolonged continuous operation.

Cultivation was carried out for the most part in a volume of 500—700 ml. The suspension was mixed by various methods: by a magnet placed in the bottom of the vessel (stirrer system), by a magnet suspended on a chain (spinner system), and by a paddle stirrer. If in the tests a unit for deep cultivation was used, then the rate of rotation of the stirrer varied from 180 to 250 rpm. The culture was aerated continuously with air or a mixture of air and carbon dioxide in set proportions with a rate of flow of gaseous mixture under the culture of 400 ml/min. The temperature was maintained constantly at around 37°. Samples were taken after specific intervals of time, pH of the suspension was determined by a change in the color of the phenol red indicator, and in necessary cases data were controlled by potentiometric indices. The amount of cells in 1 ml was calculated in a Goryayev chamber. Living and dead cells were differentiated by susceptibility to staining with Trypan Blue.

Results

For the purpose of determining the required initial parameters a number of preliminary investigations were carried out. The results of a study of the survival of FCE cells in suspension showed that under the selected conditions a sufficiently high density and viability of the culture was preserved for the first 1½ days (Fig. 3). Already in several hours after the onset of cultivation the cells had rounded off, and by 6 hours began to conglomerate, initially by 4-6-8 cells and then more, so that by 18—24 hours up to 50% of the cells were found in aggregates, the viability of which corresponded to the viability of the culture as a whole. By this time large

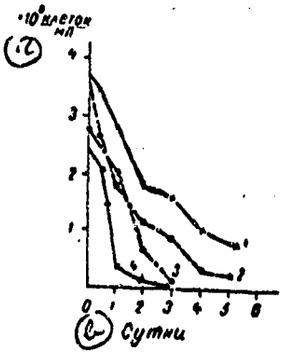

Fig. 3. Dynamics of changes in the population of FCE cells in a suspension in noninfected and VEE virus infected culture. Noninfected culture: 1 - total of cells, 2 - living, infected culture; 3 - total of cells, 4 - living.
(a) 10^6 cells/ml; (b) days.

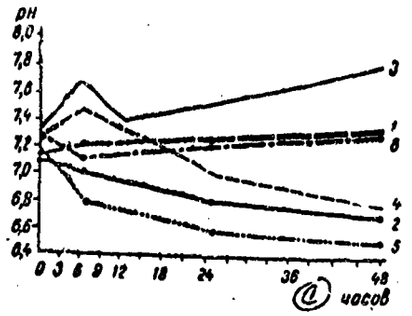

Fig. 4. Dynamics of change in the pH under various conditions of aeration.
Noninfected culture: 1 - aeration with air, 2 - air and 1.5% CO_2; infected culture: 3 - aeration with air, 4 - air and 1.5% CO_2; 5 - absence of aeration; 6 - variable regimen of aeration.
(c) hours.

solitary light cells were revealed in the culture. Formation of aggregates did not drop even with the intensive mixing of the paddle stirrer at a rate of rotation of 250—270 rpm. On the 4—5th day of cultivation the aggregates were disintegrated as a result of the death of a portion of their component cells. At this same time solitary cells began to degenerate.

Throughout the entire experiment a settling was noted of a considerable amount of cells on the walls of the culture vessel along the edge of the meniscus. The rate of formation and density of this cell ring always corresponded to the viability of the culture.

The change in the pH of the FCE culture at a density of $3.5 \cdot 10$ cells in 1 ml and aeration with air with 1.5% CO_2 is shown in Figure 4 (curves 1 and 2). Blowing through of a mixture of air with 1.5% CO_2 did not protect the culture from acidulation, and aeration with air at a rate of flow of 400 ml/min maintained the pH within optimum limits.

The stability of the virus at 37° in the medium used under conditions of constant mixing was compared with the stability of the virus in an immobile cell-less medium. The results of the experiment are shown in Fig. 5. Inactivation of the virus during mixing takes place considerably more rapidly. While in the immobile medium at 37°

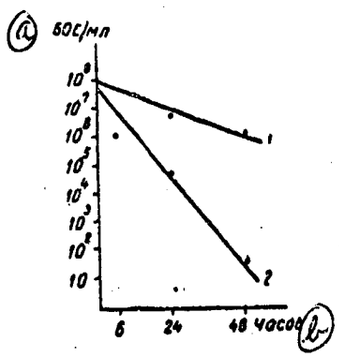

Fig. 5. Dynamics of inactivation of VEE virus in an immobile medium (1) and with mixing (2). Legend (both figures): (a) - PFU/ml; (b) - hours.

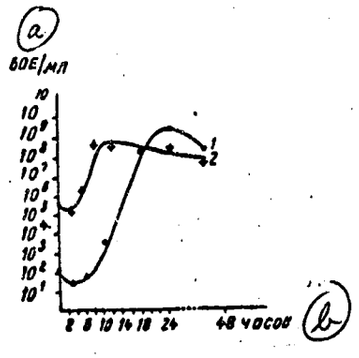

Fig. 6. Dynamics of accumulation of VEE virus in a suspension culture of FCE cells (density of population 3 • 10^5 cells in 1 ml) at a multiplicity of infection of 0.00002 PFU/cell (1) and 2 PFU/cell (2).

in 24 hours the infecting capacity of virus is reduced by 1—1.2 lg, in 24 hours with constant mixing it was reduced by 2.5—3.5 lg.

In a comparison of the various methods of mixing it turned out that when using the spinner system or the paddle stirrer the level of virus was approximately the same. As a rule the stirrer culture yielded less of an output of virus.

Dynamics of multiplication of VEE virus in a suspension of FCE cells. For studying the dynamics of accumulation of virus two parallel cultures (density of population 2.5 • 10^5 cells in 1 ml) were infected with a multiplicity of infection of 2 and 0.00002 PFU/cell (Fig. 6). A lowering of the infecting capacity of virus was noted in the first 3 hours, then the isolation of virus increased almost exponentially from 4 to 9—10 hours in the case of a high multiplicity of infection and from 6 to 22—24 hours with low multiplicity. The maximum titers in the first case comprised 4.4 • 10^8 PFU/ml, and in the second - 2.1 • 10^9 PFU/ml. In both cases in 48 hours an expressed lowering was noted in the titers of infecting capacity of the virus.

The accelerated lowering in the amount of cells in the infected culture appeared in 24 hours after the inoculation of virus, however, the viability of the cells began to drop much earlier and at considerably more expressed tempos. Figure 2 shows the dynamics of change in a culture after infection with a massive dose of virus. Aggregates, existing for a long time in an infected culture, disintegrated and died already by 24—32 hours. The cell ring on the wall of the vessel was weakly expressed and easily disintegrated. Changes, caused in a culture by infection of virus with low multiplicity, differed only by the greater lengthiness in time.

Table 1

Multiplication of the VEE virus in suspensions of chick embryo fibroblasts during various concentrations of cells and a various multiplicity of infection.

(a) Концентрация клеток (в млн/мл)	(b) Множественность инфекции (в БОЕ/клетка)	(c) Титр вируса (в БОЕ/мл) через 24 часа
1	1	$2,5 \cdot 10^7$
	0,000001	$3 \cdot 10^7$
2	5	$5 \cdot 10^8$
	0,00005	$2,1 \cdot 10^8$
5	0,1	$3,7 \cdot 10^8$
	0,00001	$8 \cdot 10^8$
7	0,4	$1 \cdot 10^9$
	0,0004	$1 \cdot 10^9$
10	2	$2,7 \cdot 10^8$
	0,0001	$4,4 \cdot 10^8$

Key: (a) Concentration of cells (in million/ml); (b) Multiplicity of infection (in PFU/cells); (c) Titer of virus (in PFU/ml) after 24 hours.

Multiplication of VEE virus in suspended cultures of FCE cells of various density. The concentration of cells varied from 1 to $10 \cdot 10^6$ cells in 1 ml. In each experiment we studied the multiplication of virus with a multiplicity of infection differing by 4—5 orders. The data obtained are cited in Table 1. The best results were achieved with moderate densities of cell population of $2—7 \cdot 10^6$ cells in 1 ml. Under these conditions the virus accumulated up to titers of $8 \cdot 10^8—2 \cdot 10^9$ PFU/ml in 24 hours. The titers of virus at a low multiplicity of infection continually turned out to be higher than during infection with a massive dose.

Comparison of productivity of VEE virus under various conditions of cultivation. Table 2 shows the maximum titers, obtained in a number of tests for a given concentration of cells for 1 ml medium, of accumulation during the application of the roller, suspension, and stationary cultivation. Stemming from the data cited the approximate harvest of virus per 1 cell was calculated. It turned out that the lowest was during its incubation in suspension, and the highest was in monolayer stationary cultures. During any method of cultivation the greatest productive capacity of virus was noted at a concentration of $1.5—3 \cdot 10^6$ cells in 1 ml of medium of accumulation.

pH of the suspension during various conditions of aeration of the culture. Figure 3 shows the results of measurements of pH of the suspension in various periods of cultivation of both infected cells and those not infected with virus. In the described series

Table 2

Harvest of VEE virus per 1 cell (FCE) under various conditions of cultivation

(a) Тип культивирования	(b) Плотность клеток на 1 мл·10⁶	(c) Максимальные титры через 24 часа (в БОЕ/мл)	(d) Урожай вируса (в БОЕ/клетка)	(e) Среднее
Стационарный монослой (f)	0,3	$4 \cdot 10^8$	1 333	
	0,5	$7 \cdot 10^8$	1 400	
	0,8	$1,3 \cdot 10^9$	1 625	1 748
	1,5	$4 \cdot 10^9$	2 633	
Роллер (g)	1,0	$7,6 \cdot 10^8$	760	
	1,5	$9 \cdot 10^8$	600	
	3,0	$6,5 \cdot 10^9$	2166	1 281
	5,0	$8 \cdot 10^9$	1600	
(h) Суспензия	2,0	$2,1 \cdot 10^8$	1 050	
	5,0	$8 \cdot 10^8$	160	
	7,0	$1 \cdot 10^9$	143	349
	10,0	$4,4 \cdot 10^9$	44	

Key: (a) Type of cultivation; (b) Density of cells per 1 ml $\cdot 10^6$; (c) Maximum titers in 24 hours (in PFU/ml); (d) Harvest of virus (in PFU/cell); (e) Average; (f) Stationary monolayer; (g) Roller; (h) Suspension.

of tests the density of population comprised $3 \cdot 10^6$ cells in 1 ml, multiplicity of infection - 2 PFU/ cell, and mixing and aeration were in conformity with the regimen described above. While the blowing through of pure air maintained the pH of a noninfected suspension under optimum limits (1), the presence of 1.5% of CO_2 in a gaseous mixture quite rapidly led to a lowering of the pH to 6.8—6.7 (2). In the absence of aeration an infected culture already in several hours produced intensive acidulation of the medium (3). In the case of blowing through of pure air through an infected culture (3) the pH of the suspension in the course of 6 hours of incubation increased gradually by 0.3—0.4; the phase of logarithmic growth in the isolation of virus was connected with a noticeable acidulation of the medium. Subsequently as a result of the death of cells the pH was established on a quite high level. Blowing through of a 1.5% CO_2 suspension somewhat decreased the initial alkalization of the culture, but during prolonged cultivation the pH was reduced to 6.8. A change in the concentration of carbon dioxide in the gaseous mixture - 3,5% CO_2 during the first 6 hours, air up to 24 hours, and, if further maintenance of the culture was necessary, 1.5% CO_2, made it possible to maintain the pH in satisfactory limits without sharp fluctuations.

Discussion

The multiplication of VEE virus in suspensions of trypsinized PCE cells is subordinated to the same general principles which are characteristic for its development in monolayer cultures of these cells. The use of a suspension culture makes it possible to achieve a considerable economy of time, nutrient media, and labor expenditure. Since the greatest density and viability of cell population is noted in the first hours of cultivation, then this method can be used with great success for the incubation of viruses with a short cycle of development. By multiplying the virus considerably speeds up the death of the culture and curtails the process of survival of cells to several hours. The higher the multiplicity of infection the more significant this is.

The influence of multiplicity of infection on the maximum titers of virus apparently has the same mechanism which was noted by us for roller cultures, but less expressed. It is necessary to add that with a low multiplicity of infection the cells in 10—14 hours are able to become somewhat adapted to existence in a suspension and increase the production of virus. A certain increase in the yield of virus following infection with small doses in suspensions of trypsinized cells has been described for the virus of fowl plague (6) and poliomyelitis [3]. As a rule for suspension cultures a considerable scattering of data and a certain inconstancy of results are characteristic for surviving cells.

The fact that, based on yield of virus for 1 cell, suspension cultures give in considerably to the method of monolayer cultures has been noted many times by many authors on various cell and virus models [5, 7, 8, 10, 13, 14, 17]. From our point of view it is insufficient to explain this phenomenon just by the physiological state of cells in suspension, thus conditioning a lesser production of virus by each cell. In suspensions of chick fibroblasts even when using a large multiplicity of infection a considerable amount of noninfected cells remain [9]. On the other hand destruction of the virus in a suspension proceeds very intensively. Finally, the method of cultivation of viruses in suspensions of trypsinized cells is not near as developed as the method of their multiplication in monolayer cultures.

One of the most important factors which determine the success of suspension cultivation is the maintenance of optimum pH values. Acidulation of the medium sharply speeds up the death of virions, meanwhile a shift of pH to the acid side increases by 2—4 times the activity of inhibitors of bovine serum. A correct regimen of aeration makes it possible to simply and effectively regulate the reaction of the medium. There is interest in the nature of the curve for change of pH during aeration of a culture with air (see Fig. 3, curve 3), which is apparently connected with the cycle of

reproduction of the virus. The initial overalkalinity of the medium was probably conditioned by the active removal, by the stream of air, of the volatile products of cell metabolism (CO_2 in particular) which were formed as a result of the activation of energy processes of the cells under the influence of virus infection. A subsequent lowering of pH takes place due to the greater accumulation of nonvolatile acid substances. It is mainly their liberation which hampers the maintenance of pH by the method of aeration with high concentrations of cells.

We propose the following optimum method for the accelerated and economical obtaining of VEE virus in the unit for deep cultivation. FCE cells, obtained by means of trypsinization of 10—11 day chick embryos are washed once with Hanks solution, they are diluted in a mixture of medium No 199 and 0.5% solution of lactalbumin hydrolyzate (1:1) to a density of 3—3.5 • 10^6 cells in 1 ml, and 5—7% of heated bovine serum and antibiotics (100 AU/ml each of penicillin and streptomycin) are added. The suspension of cells is placed in a culture vessel and the virus is introduced on the basis of 10^{-4} and 10^{-5} PFU/cell. The temperature of incubation is set at 37°C, rate of rotation of the stirrer is around 200 rpm, and flow of gaseous mixture is 400 ml/min; the pH is maintained by the corresponding regimen of aeration, which is selected depending on conditions; gassing for 24 hours with 1.5% CO_2 or gassing for the first 6 hours with 3.5—4% CO_2 with subsequent blowing through of air for up to 24 hours. During cultivation in closed vessels without aeration (spinner system for example) it is necessary to select the correct ratio of liquid and gaseous phases (1:10—1:20) or to use cotton plugs, add soda periodically, etc. In 24 hours the culture is removed and the suspension centrifuged at 6,000 g for 15 min. Infectious titers of virus and supernatant liquid reach 10^9 PFU/ml. The entire process of production of virus occupies 26—28 hours.

Conclusions

1. The unit for deep cultivation can be used successfully for the multiplication of viruses in a suspension of trypsinized cells.

2. It ~~has been~~ demonstrated that the effectiveness of reproduction of virus is determined by the multiplicity of infection, concentration of cells in the suspension, and the regimen of aeration.

3. Optimum technological conditions have been developed for the accelerated obtaining of large quantities of a representative of group A arboviruses - the VEE virus.

Literature

1. Vasil'yeva, O. A., Mekhanikova, V. G., In the book: Tick-borne Encephalitis, Leptospirosis, Listeriosis, Toxoplasmosis., Tomsk, 1963, p 4.
2. Kokorev, V. S., In the book: Inhibitors of Virus Activity,

Riga, 1967, p 47.
3. Mironova, L. L., Elbert, L. B., In the book: Poliomyelitis and Other Enterovirus Infections, Moscow, 1963, p 224.
4. Pozdnyakov, A. A., Sergeyev, V. A., Vopr. virusol., 1965, No 3, p 338.
5. Pozdnyakov, A. A., Sushkov, F. V., Sergeyev, V. A., et al., In the book: Problems of Veterinary Virology, Moscow, 1966, vol 2, p 325.
6. Sergeyev, V. A., Khizhinskaya, V. P., Veterinariya, 1965, No 2, p 18.
7. Bachrach, H. L., Callis, J. J., Hess, W. R., Proc. Soc. exp. Biol. (N.Y.), 1956, v 91, p 177.
8. Dulbecco, R., Vogt, M., J. exp. Med., 1954, v 99, p 183.
9. Gramenti, F., Rossi, G., Vet. ital., 1962, v 13, p 576.
10. Hardy, F. M., Brown, A., J. Bact., 1961, v 82, p 449.
11. Mazzaracchio, V., Orfei, Z., Tiecco, G., et al., Zooprofilassi, 1957, v 12, p 539.
12. Mayer, V., Zemla, J., Acta virol., 1962, v 6, p 53.
13. Mussgay, M., Zbl. Bakt., 1 Abt. Orig., 1960, Bd 177, p 437.
14. Patty, R. E., Bachrach, H. L., Hess, W. R., Am. J. vet. Res., 1960, v 21, p 144.
15. Pereira, H. G., J. Path. Bact., 1953, v 65, p 259.
16. Idem, J. gen. Microbiol., 1954, v 10, p 500.
17. Rubin, H., Baluda, M., Hotchin, J. E., J. exp. Med., 1955, v 101, p 205.